Gonadal Asymmetry and Sex Determination in Birds

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Gonadal Asymmetry and Sex Determination in Birds

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Abstract
Although vertebrates display a superficial bilateral symmetry, most internal organs develop and locate with a consistent left:right asymmetry. There is still considerable debate as to when this process actually begins, but it seems that, at least for some species, the initial steps occur at a very early stage of development. In recent years, a number of model systems, including the chick embryo, have been utilised to increase our understanding of the molecular basis of this complex developmental process. While the basic elements of asymmetry are clearly conserved in chick development, the chick embryo also exhibits an additional unusual asymmetry in terms of the development of the gonads. In the female chick embryo, only 1 gonad and accessory structures fully develop, with the result that the adult hen has only 1 ovary and a single oviduct – both on the left side. With a small number of exceptions, this is a consistent feature of avian development. Here, we describe the morphological development and molecular basis of this unusual asymmetry, consider the implications for avian sex determination, and discuss the possible biological reasons why many birds have adopted a single-ovary system.

Vertebrates display a superficial bilateral symmetry, a basic body plan in which the left and right sides form mirror images across the midline, but internally most organs develop and locate asymmetrically [Wolpert, 2005]. These include the heart, spleen and stomach, and also bilateral organs such as lungs and kidneys which differ in appearance or position on the right and left sides. Lateralisation also affects the structure and the function of the brain hemispheres, generating a spectrum of asymmetric behaviours specific to different vertebrates (e.g. handedness in humans) [Vandenberg and Levin, 2013]. To add to the complexity, some tissues which develop symmetrically, such as the somites, do so despite the expression of left:right (L:R) determinants, because the asymmetry is overridden by retinoic acid (RA) signalling [Vermot and Pourquie, 2005; Duester, 2007; Vilhais-Neto et al., 2010].

In recent years, enormous progress has been made in understanding the molecular and developmental basis of

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L:R asymmetry. This complex process involves multiple steps including: orientation of the cytoskeleton, the redistribution of morphogens, L:R transcription of asymmetry genes and, finally, asymmetric organogenesis [Levin, 2005; Raya and Izpisua Belmonte, 2006; Vandenberg and Levin, 2013]. There is considerable debate as to when this process begins. If the anterior:posterior and dorsal:ventral axes are apparent, it is possible to say which side of an embryo will be left or right, but markers of L:R asymmetry could develop independently and precede or follow these other axes. It is often assumed that the initial steps occur at gastrulation/node formation; however, it seems that, at least for some species, the first steps occur at a much earlier stage of development [Vandenberg and Levin, 2013].

The chick embryo is amongst a number of model systems that have been utilised to establish our basic understanding of L:R asymmetry in vertebrates. While the basic elements are clearly conserved in this model, the chick also exhibits an unusual asymmetry in terms of the development of the gonads. This is most obviously manifested in the adult female that has only a single ova-producing ovary and a single oviduct, both originating from primordia on the left side of the embryo. Although this condition is frequently ascribed to 'birds' en masse, and is true for most birds, species from at least 16 of the >35 orders of birds do have 2 ovaries [Gunn, 1912; Shaw, 1938; Kinsky, 1971].

Here, we describe in detail the L:R morphological development of the embryonic chick gonads, consider the molecular basis of this unusual asymmetry, and discuss the possible biological reasons why many birds have adopted a single-ovary system.

Morphology

In chick embryos, as in mammalian embryos, the gonads arise as a thickening of the coelomic epithelium which forms a ridge running in an anterior:posterior orientation on the ventral surface of each mesonephros (primitive kidney). Gonadogenesis begins at around 72 h of development (Hamburger Hamilton (HH) developmental stage 23) [Hamburger and Hamilton, 1951], but the genital ridges are not macroscopically evident until HH26 when both are approximately 1.5 mm in length and 0.1 mm wide (fig. 1a) [Swift, 1915; Romanoff, 1960; Carlon and Stahl, 1985]. As development proceeds, the morphological appearance of the gonads is initially similar in males and females (HH28), but by HH36, the gonads in males and females are distinctly different. The right and left testes become tubular structures, approximately 3 mm long and 0.5 mm wide, and while the right female gonad is similar in appearance but slightly smaller than the testes in males, the left female gonad has acquired a broader, flatter appearance and is markedly larger at approximately 3.2 mm long and 0.8 mm wide (fig. 1b–h). Throughout embryonic development, male and female gonads continue to increase in size and immediately prior to hatching both testes are approximately 5 mm long and 1.5 mm wide, while in the female the right gonad is around 2.6 mm by 0.5 mm and the left, which is clearly an ovary, is 8 mm by 1.5 mm. The physical growth of the gonads between HH36 and HH44 is reflected by increases in wet weight and RNA content (fig. 1i–l).

In the initial stages of gonadogenesis, the epithelial ridge is composed of columnar cells that overlie tissue that lacks the typical nephron structure characteristic of the underlying mesonephros and that is instead composed of clusters of epithelial-like cells embedded in mesenchyme [Carlon and Stahl, 1985]. By HH25, the 'genital ridges' protrude into the coelomic cavity as distinct organs and comprise a pseudo-stratified columnar epithelium (the germinal epithelium) covering a central core (the medulla) organised into epithelial cords. The origin of these so-called primitive sex cords, which are also apparent in some mammals such as humans, but not the mouse, is still unclear. Some studies indicate the germinal epithelium as a source, while others suggest that they are of mesonephric origin [Stahl and Carlon, 1973; Merchant-Larios et al., 1984; Rodemer-Lenz, 1989; Sekido and Lovell-Badge, 2007]. At this point, both male and female gonads already display a L:R morphological asymmetry, with the epithelial layer on the left gonad thicker than that on the right [Carlon and Stahl, 1985] (fig. 2). This asymmetric feature is maintained in both sexes at the time of gonadal sex determination (thought to be around HH27–28). By HH29, the epithelium surrounding the right gonad flattens and adopts a more squamous-like appearance, further emphasising the L:R asymmetry in both sexes [Carlon and Stahl, 1985]. As gonadal sex differentiation progresses, the morphological differences between left and right gonads in the female become more pronounced, while the asymmetry between male gonads diminishes (fig. 1c–h, 2). By HH36, both left and right testes have a similar organisation: a thin flat simple epithelium overlaying a core organised into well-defined branched tubular structures. These branched structures are designated sex cords or testis cords and comprise germ cells and differentiating Sertoli cells encased within

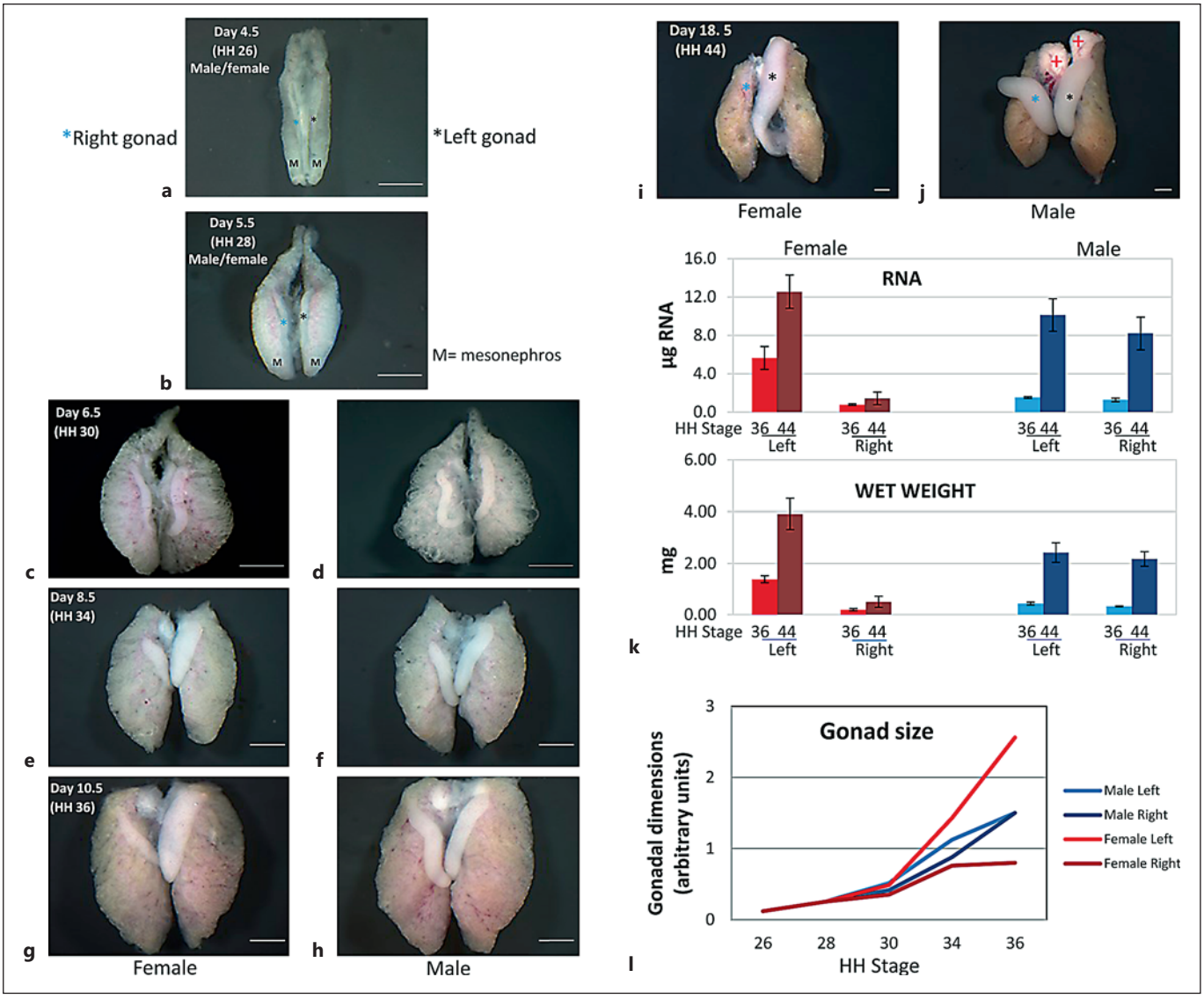


Fig. 1. Gross morphology of the developing chick gonads. **a–j** Ventral view of gonads and mesonephroi from day 4.5 (HH26) to day 18.5 (HH44) of development. Left and right gonads are indicated by black and blue asterisks, respectively. Left gonads are larger than right gonads in both sexes in the early period (**b–f**). In the later stages of development, the left gonad is larger than the right only in females (**g–j**). Adrenal glands (red +) are clearly visible in the later developmental stages. Size of bars = 1 mm. **k** Total RNA content and wet weight of male and female left and right gonads increase between day 10.5 (HH36) and day 18.5 (HH44). Histograms represent mean values of at least 3 pools of 10 individual gonads. **l** Graph illustrating changes in gonadal size between day 4.5 (HH26) and day 10.5 (HH36) of embryonic development. Approximate gonadal dimensions are calculated on the basis of the length and width at mid-point of individual gonads (L × W).

a basement membrane (fig. 3b–d). In contrast, the overall structure of left and right ovaries is clearly different and these differences are largely confined to the outer epithelial layer (fig. 3a). While the right ovary, like the testes, is surrounded by a thin flat simple epithelial layer, the left ovary is enclosed in a thick sheath of stratified epithelial-like cells, known as the cortex (fig. 2, 3a) [Romanoff, 1960]. The medulla has a similar organisation in both ovaries with irregular disorganised cords of epithelial-like cells and vesicular structures known as lacunae. By HH39, most of the cells of the cortex are organised into cord structures around groups of germ cells and these 'secondary' cords extend into the medullary region [Gonzalez-Moran, 2011].

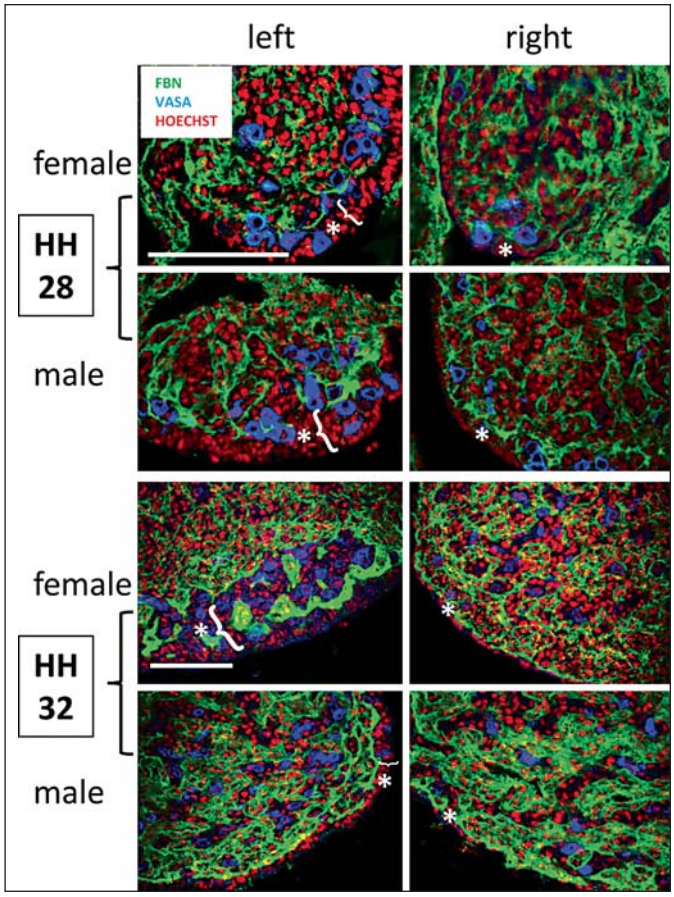


Fig. 2. Development of gonadal epithelium in embryonic chick gonads. Sections through female and male chick gonads immunostained for fibronectin (FBN) and the germ cell marker VASA, and stained with the nuclear marker Hoechst. Images illustrate the relative thickness of the surface epithelial layer in left and right, male and female gonads at day 5.5 (HH28) and day 7.5 (HH32) of development. The epithelial layer is thicker on the left than on the right gonad in both sexes at the stages illustrated. Cortex formation is obvious in the left female gonad at HH32. Location of epithelial layers is indicated by position of the asterisk (*) and approximate thickness is indicated by the size of the bracket ({). Size of bars = 100 μm.

The L:R asymmetry associated with the embryonic gonads is not confined to the somatic component. There is also a clear L:R asymmetry in the distribution of the germ cells (fig. 4). As early as HH15, the number of primordial germ cells (PGC) in the intermediate mesoderm in the area beneath the forming ridges, was found to be consistently and significantly higher on the left than on the right in both sexes with an approximately 3:2 L:R split by HH17 [Nakamura et al., 2007]. This trend persists and accentuates during the colonisation of the genital ridge so that in both sexes, the left gonad contains more germ cells than the right (fig. 4a). This asymmetry has been observed in the chick and other birds (e.g. quail and duck) [Van Limborgh, 1968; Didier and Fargeix, 1976; Bergeaud et al., 1977; Dubois and Cuminge, 1978; Nakamura et al., 2007; Intarapat and Stern, 2013]. There is evidence that in the chick, as in the mouse, PGC colonisation of the gonads is driven by chemoattractants secreted by the gonadal mesoderm [Molyneaux et al., 2003; Stebler et al., 2004]. So it is possible that the unequal L:R distribution of germ cells is simply due to a L:R asymmetry in the level of gonadal chemokines – a hypothesis first suggested by very early studies on germ cell migration [Witschi, 1935; Baillie et al., 1966; Dubois, 1968]. Interestingly, it has been reported that between HH22 and HH26 in chick, well before gonadal sex determination, this asymmetry is more pronounced in females than in males with an approximately 4:1 and 2:1 L:R split, respectively, indicating that at least some sex-specific differences are present before the accepted time of sex determination [Van Limborgh, 1968]. Moreover, unlike the situation in mammals, the germ cells are not initially distributed throughout the genital ridges, but are mostly localised close to the epithelium in the left and in the right gonad in both females and males (around 70–85%) [Van Limborgh, 1968]. However, by HH30 this situation persists only in the left female gonad, while the germ cells become randomly distributed throughout the right female gonad and throughout both left and right male gonads (fig. 4a).

This particular asymmetry is maintained into the later stages of ovary development (HH44) so that in the left ovary, the germ cells are found predominately in the cortex, and in the right ovary, a smaller number of germ cells appear to be randomly distributed throughout the core region. In the male, similar numbers of germ cells are located in the sex cords of both right and left testes (fig. 4b).

A number of studies have followed the fate of germ cells in the period immediately prior to hatching. It has been proposed that the germ cells of the right embryonic ovary are lost by cell death or cell abandonment based on the identification of apoptotic germ cells and of germ cells within the lacunae [Ukeshima and Fujimoto, 1991; Ukeshima, 1994, 1996]. However, a more recent study [Gonzalez-Moran, 2011] reports that the total number of germ cells in the right ovary increases up to the point of hatch, suggesting that any loss is compensated for by germ cell proliferation. The same study found that the number of germ cells in the medulla of the left ovary decreases after HH38–39 indicating a progressive elimination during the second half of embryogenesis [Gonzalez-Moran, 2011]. It

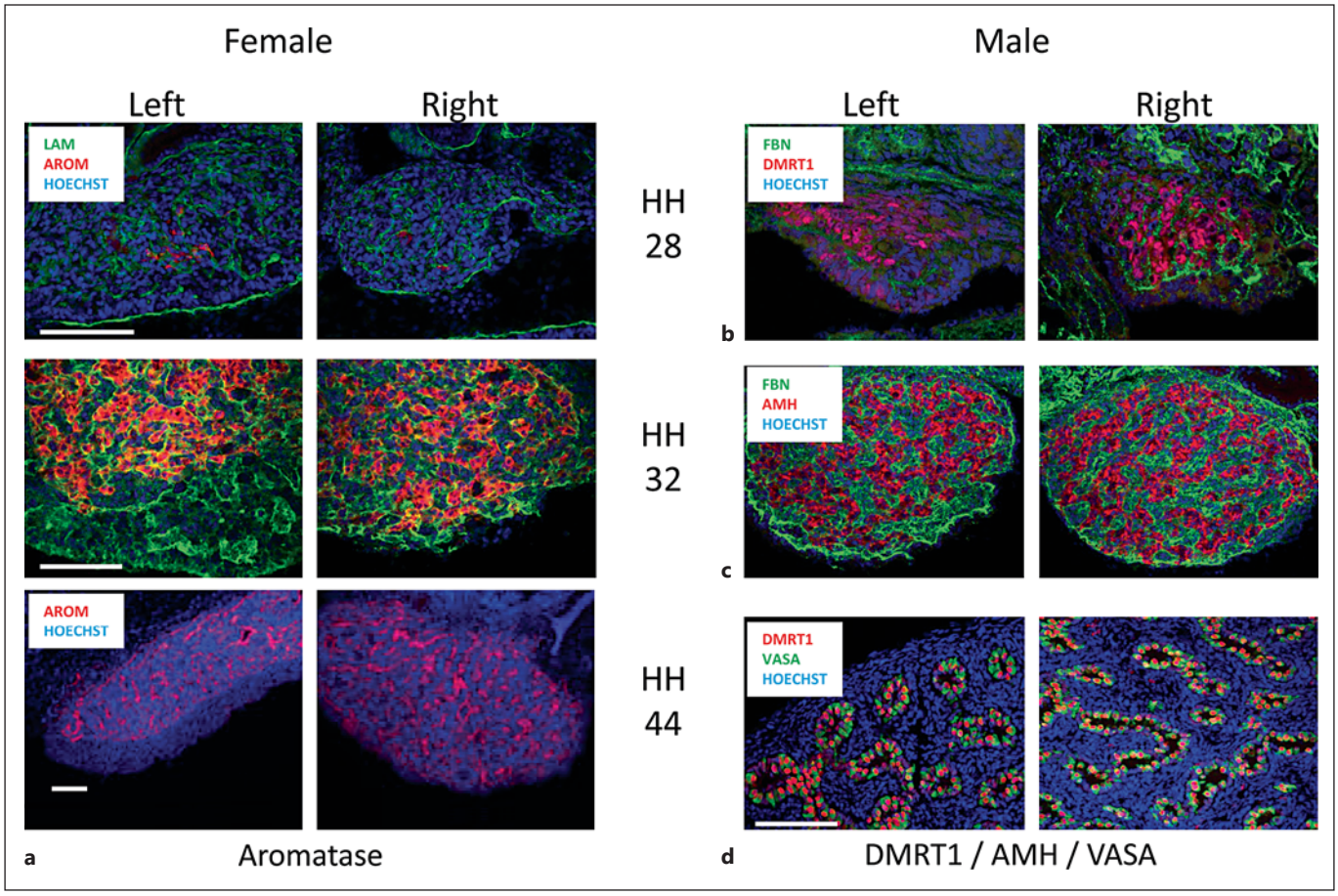


Fig. 3. Sex-specific differentiation of the embryonic chick gonads. Sections through female and male chick gonads immunostained for sexually dimorphic markers and stained with the nuclear marker Hoechst. **a** Female chick gonads between day 5.5 (HH28) and 18.5 (HH44) of development immunostained for laminin (LAM) and aromatase (AROM). Strong expression of aromatase is evident in the medullary region of both left and right gonads at day 7.5 (HH32) and is maintained through day 18.5 (HH44). An obvious cortex is present in the left ovary by stage 32. **b** Male chick gonads immunostained for DMRT1 and FBN. **c** Male chick gonads immunostained for anti-Müllerian hormone (AMH) and FBN. Images illustrate the formation of sex cords and the acquisition of sexually dimorphic function (AMH synthesis). **d** Male chick gonads immunostained for DMRT1 and the germ cell marker VASA. Images illustrate germ cell-filled testis cords at day 18.5 (HH44). DMRT1 is expressed in the somatic compartment of the developing gonad at day 5.5 (**b**), and while DMRT1 is also expressed in the Sertoli cells at day 18.5 (**d**), expression in the later stages is predominately in the germ cells. Size of bars = 100 μm.

is also noteworthy that, by HH41–42, most of the cortical germ cells of the left ovary have entered meiosis [Hughes, 1963; Ukeshima and Fujimoto, 1991; Smith et al., 2008a; Yu et al., 2013]. At this time, medullary germ cells of both female gonads do not possess the typical morphology of meiotic cells, nor do they express typical leptotene proteins, such as SCP3 [Ukeshima and Fujimoto, 1991; Smith et al., 2008a], suggesting that germ cells in the medulla either never enter meiosis, or are delayed in doing so. By 4 weeks post-hatch, the medulla in both left and right female gonads has lost all germ cells and is organised into large compacted cords with much reduced lacunar channels [Gonzalez-Moran, 2011].

The right female gonad does not develop into a functional ovarian structure in the adult and is considered to eventually atrophy. However, the removal of the left ovary in the young chick results in the differentiation of the right gonad into a testis-like organ [Groenendijk-Huijbers, 1965, 1967], indicating that the right gonad is not completely lost, and there is evidence suggesting that the right ovary can maintain a steroidogenic function [Narbaitz and Kolodny, 1964; Samar et al., 1983].

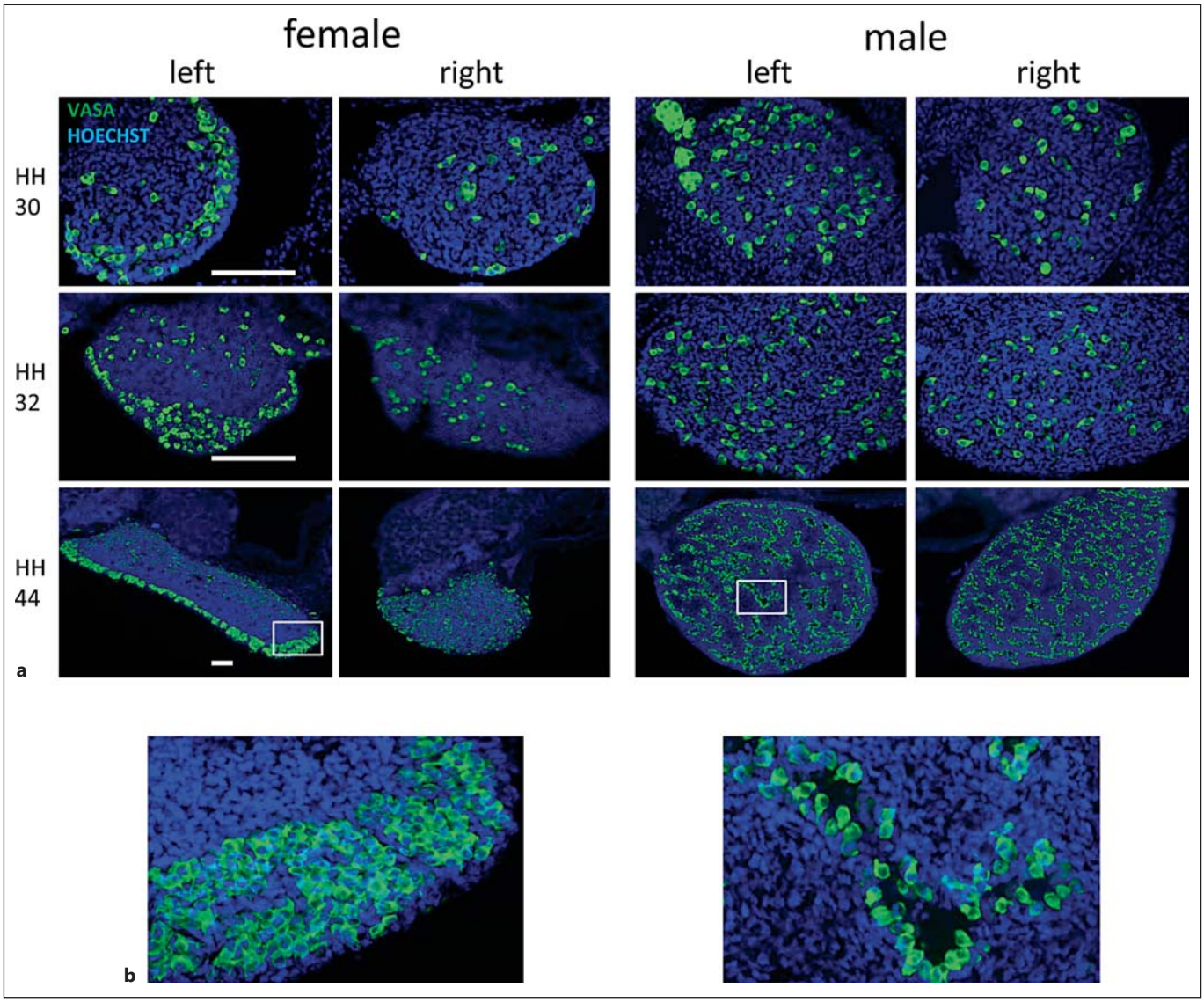


Fig. 4. Distribution of germ cells in the left and right gonads of female and male chick embryos. **a**, **b** Sections through female and male chick gonads immunostained for the germ cell marker VASA and stained with the nuclear marker Hoechst. **a** Localisation of germ cells in female and male gonads at day 6.5 (HH30), day 7.5 (HH32) and day 18.5 (HH44). In the earlier stages, greater numbers of germ cells are present in the left gonad than in the right gonad in both males and females. This asymmetric pattern of germ cell distribution is maintained in the later stages of embryonic development in the female gonads (HH44), but not in the male. Images illustrate cortical distribution of germ cells in the left female gonad, the random distribution of germ cells in the right female gonad, and the presence of germ cells in a cord-like arrangement in both male testes. **b** Higher-magnification images of areas framed in **a** (HH44). Size of bars = 100 μm.

In contrast, the left ovary becomes a fully functional reproductive organ similar in structure to the ovary of all other vertebrates: a central medulla mainly composed of stromal-vascular tissue and a cortex containing meiotic oocytes surrounded by granulosa cells and steroidogenic cells [DeFalco and Capel, 2009].

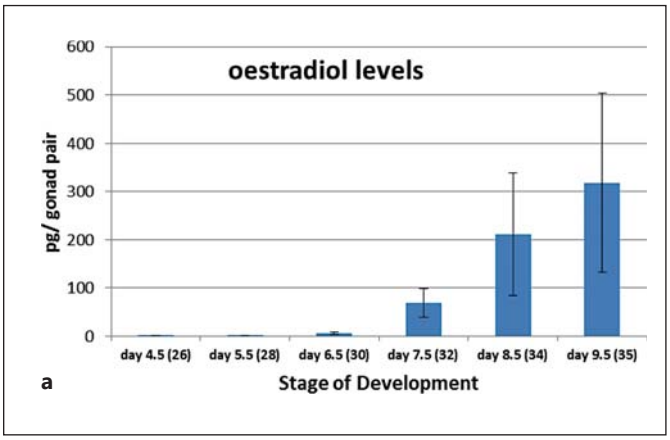
Molecular Asymmetry

Analysis of cell growth at HH27–29 showed that the proliferation rate in the epithelium of the left gonad is higher than that found in the epithelium of the right gonad in both male and female embryos, while the prolif-

eration rate in the right and left medulla of both sexes was similar [Ishimaru et al., 2008]. It is possible that this differential proliferation is due, at least partially, to steroidogenic factor 1 (SF1). This transcription factor is expressed in the medulla of left and right genital ridges, but only in the left epithelium. The finding that SF1 up-regulates the expression of the cell cycle regulator cyclin D1 (CD1) and increases cell proliferation in the epithelium led to the proposal that SF1 may directly activate the *CD1* promoter, perhaps through interaction with β-catenin [Ishimaru et al., 2008] via a similar mechanism to that seen with *CD1* activation by the *SF1* homologous gene *LRH1* [Botrugno et al., 2004]. However, *CD1* activation alone cannot account for ovarian asymmetry: while *CD1* activation in the right epithelium led to an increase in overall gonadal size, it was not sufficient to induce cortical differentiation [Ishimaru et al., 2008; Rodriguez-Leon et al., 2008].

Cells of the gonad epithelium have also been shown to display an asymmetry in relation to the orientation of the plane of division [Rodriguez-Leon et al., 2008]. In a typical epithelial monolayer, cell division can be either perpendicular or parallel to the epithelial plane. With the former, both cells remain in the epithelial layer and this is described as a symmetrical division, while the latter may result in 1 daughter cell leaving the monolayer leading to an asymmetric division [Betschinger and Knoblich, 2004; Woolner and Papalopulu, 2012]. At HH27, the percentage of potential asymmetric cell divisions in the epithelium of the left gonad is almost twice that found in the epithelium of the right gonad [Rodriguez-Leon et al., 2008]. While the significance of this finding is not certain, it is known that symmetrical divisions result in epithelial growth, whereas asymmetric divisions can generate stratified epithelia, and/or produce new cell types [Baena-Lopez et al., 2005; Woolner and Papalopulu, 2012]. It is possible that the combination of a higher proliferation rate and a bias in favour of asymmetric cell division on the left side may contribute to the patterning of the cortex.

Not surprisingly given the morphological differences between the left and right epithelia, several cell adhesion and extracellular matrix components have also been shown to display L:R asymmetry [Guioli and Lovell-Badge, 2007; Rodriguez-Leon et al., 2008]. These include N-cadherin and cytoplasmic β-catenin which show distinct subcellular localisation in left and right epithelia from HH28 [Rodriguez-Leon et al., 2008]. Moreover, the basal lamina beneath the epithelium acquires a different structure on the left compared to the right by HH28. Indeed it appears more discontinuous and enriched in fibronectin (fig. 2) [Guioli and Lovell-Badge, 2007]. Adhesion properties are obviously important determinants in various aspects of epithelial morphogenesis, including spindle orientation, cell shape and cell migration, and are therefore potential players in the fate of left and right epithelia [Nelson, 2009; Cai and Mostov, 2012].



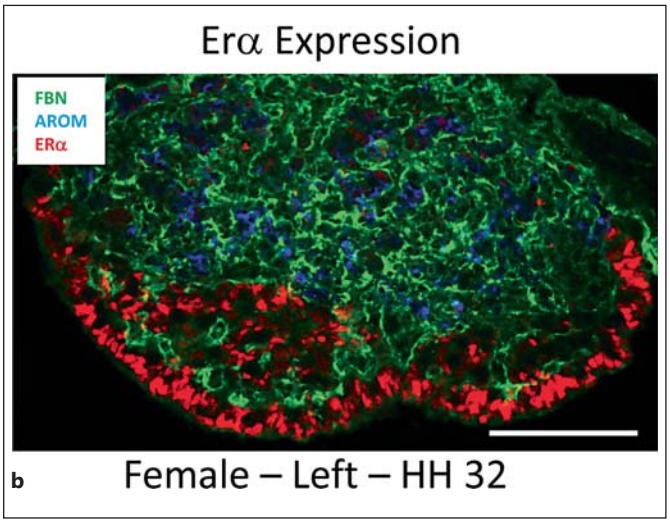


Fig. 5. Estrogen synthesis in embryonic chick gonads. **a** Graph depicting 17β-oestradiol synthesis in the female chick gonads between day 4.5 (HH26) and day 9.5 (HH35) of embryonic development. Histograms represent the mean oestradiol level per gonad pair. Values were calculated from measurement of 17β-oestradiol content of 4 pools of at least 10 gonads each. **b** Section through day 7.5 (HH32) female left gonad immunostained for ERα, aromatase (AROM) and fibronectin (FBN), illustrating nuclear expression of ERα. Size of bar = 100 μm.

In the female chick embryo, both the left and the right gonadal medulla synthesise estrogen from HH29–30 onwards, as assessed by expression of P450 aromatase (the enzyme that catalyses the conversion of androgens into estrogens) (fig. 5a) [Andrews et al., 1997; Nakabayashi et

al., 1998]. This steroid hormone modulates gene transcription and activates cell signalling, mostly via binding to estrogen receptors (ERs) [Marino et al., 2006]. The only ER found to be expressed in the embryonic gonad is ERα, and this receptor shows an asymmetric expression pattern as early as HH25–26 in both sexes [Andrews et al., 1997; Nakabayashi et al., 1998]. Epithelial expression of ERα is detected only in the left gonad, although medullary expression is found in both left and right gonads. Crucially, although this pattern of RNA transcription is similar in both males and females, the translated protein displays a sexually dimorphic profile. Immunohistochemistry studies found conspicuous amounts of nuclear ERα protein reflecting the RNA pattern only in the female (fig. 5b), while in the male the protein was barely detectable and cytoplasmic [Andrews et al., 1997; Guioli and Lovell-Badge, 2007]. This female-specific asymmetric ERα pattern was shown to persist at least up to HH39 [Guioli and Lovell-Badge, 2007], and it coincides with the distribution pattern of estrogen target cells defined by radio-immuno assays [Gasc, 1980]. As estrogen is an essential element for ovarian determination in birds (see next chapter), ERα asymmetry may be a primary cause of the different fates of the left and right ovaries.

Because both male and female gonads clearly show distinct L:R morphological and molecular features prior to the appearance of any obvious sexual differentiation, a number of studies focused on the signalling pathway that controls the L:R body axis. Following gastrulation, this pathway operates through a cascade of asymmetric molecular signals initiated at the node and propagated to the lateral plate mesoderm. The downstream target on the left side is the transcription factor *PITX2* [Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Gage et al., 1999]. It was found that this gene is expressed exclusively in the epithelium of the left gonad in both sexes from the time the gonadal primordia arise, and that this is maintained at least until HH39 [Guioli and Lovell-Badge, 2007]. When visceral organ heterotaxia was induced using the pharmacological compound Lindane, it was found that gonad situs-specific morphogenesis was also affected [Guioli and Lovell-Badge, 2007]. Moreover, it was shown that the interference of the L:R pathway is mediated via a direct instructive role of *PITX2* at organ level, as *PITX2* expression in the right gonad epithelium was sufficient to induce the differentiation of a cortex containing meiotic germ cells [Guioli and Lovell-Badge, 2007] (fig. 6). Two further studies confirmed the role of *PITX2* in conferring epithelial left identity [Ishimaru et al., 2008; Rodriguez-Leon et al., 2008].

All the L:R molecular differences identified to date are limited to the epithelium and are downstream of *PITX2*, indicating that *PITX2* directly or indirectly affects multiple aspects of gonad epithelial morphogenesis, including the proliferation, adhesion and estrogen signalling properties already described [Guioli and Lovell-Badge, 2007; Ishimaru et al., 2008; Rodriguez-Leon et al., 2008].

Specific details of the molecular mechanisms by which PITX2 may control differentiation of the cortex are still poorly understood. It has been shown that components of RA signalling are affected by the gonadal asymmetry established by *PITX2*, including nuclear receptors activated upon RA binding (RARα and RXRα) and enzymes that are involved in the synthesis and breakdown of RA (RALDH2 and CYP26A1, respectively) [Ishimaru et al., 2008]. By HH27, epithelial RARα and RXRα levels are higher in the right than in the left gonad suggesting that RA signalling is activated preferentially in the right epithelium. This is supported by the fact that *RALDH2* and *CYP26A1* show a complementary expression pattern with respect to the gonadal epithelia: with *RALDH2* expressed on the right and *CYP26A1* expressed on the left. A series of experiments were carried out where beads soaked in RA were implanted in the left side of the embryo. RA did not affect *PITX2* expression in the epithelium of the left gonad, but it did down-regulate the expression of *ERα, SF1* and *CD1* and decreased the rate of proliferation in the epithelium. Conversely, *ERα, SF1* and *CD1* expression was stimulated in the epithelium of the right gonad by an RA-antagonist [Ishimaru et al., 2008]. The treatments had no obvious effect on medullary tissue. These data suggest that down-regulation of RA signalling within the epithelium allows the epithelium to acquire 'left identity', based on the markers analysed. However, this study was performed only up to the widely accepted point of 'gonadal sex determination' (HH27–28); therefore, it is not certain that the induced 'left identity' would be maintained on the RA–ve/PITX2–ve right side, or if the potential loss of left identity persists on the RA+ve/PITX2+ve left side, as the formation/maintenance of a proper cortex was not assessed. Interestingly, in the period just prior to the expression of the meiotic gene *STRA8* and the initiation of meiosis (HH38–41), the somatic cells of the left cortex do express *RALDH2* and do not express any *CYP26*, suggesting that in chick, as in mammals, RA is important for the initiation of meiosis [Smith et al., 2008a; Yu et al., 2013]. At this stage of development, although *PITX2* is still expressed, it is down-regulated in comparison to earlier stages [S.G., unpubl.

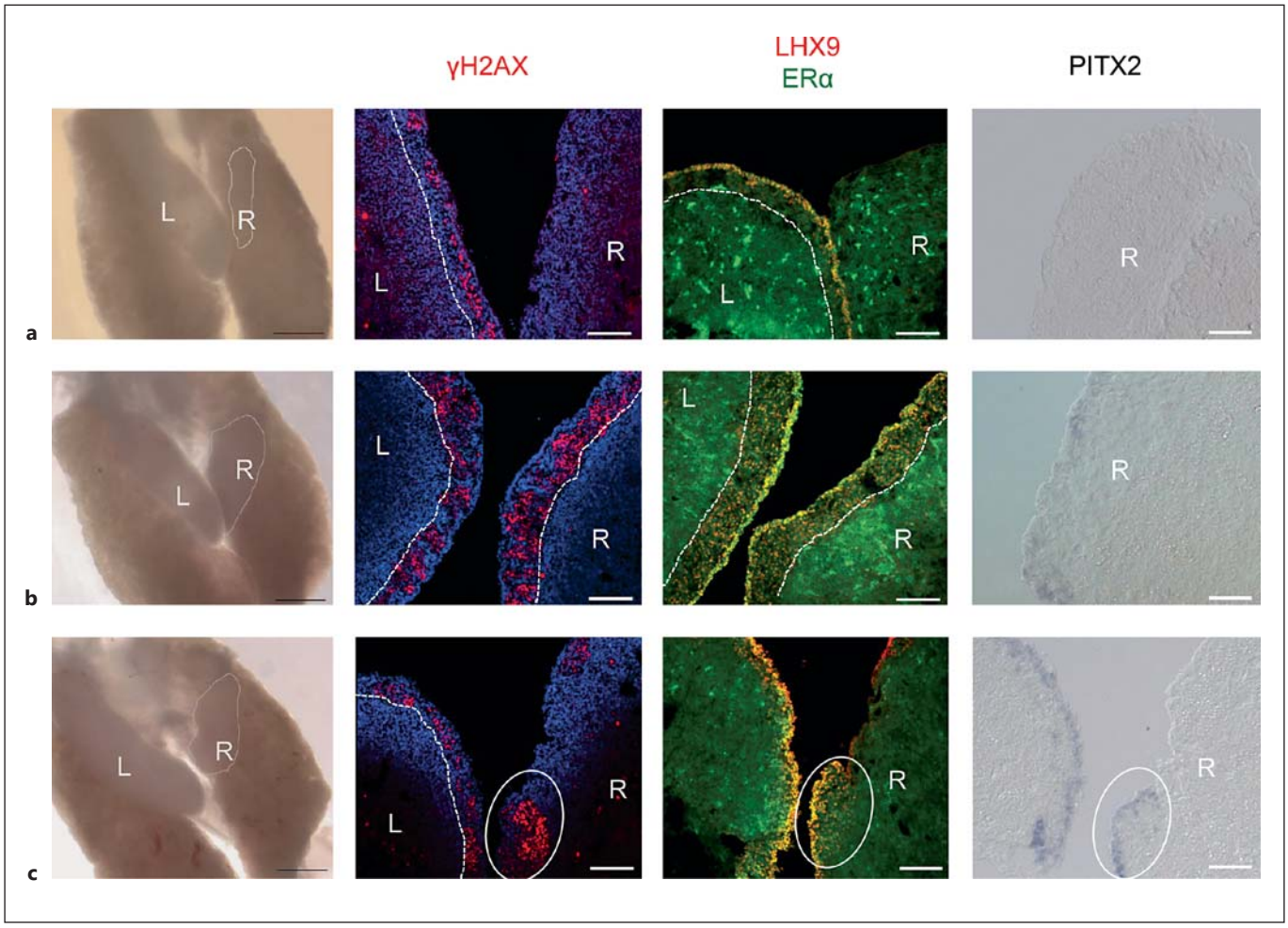


Fig. 6. PITX2 confers left identity to the differentiating ovary. E12–13 (HH38–39) gonads from embryos infected in ovo with a RCAS-PITX2a virus injected at HH8–10 in the posterior right side. Sections were analysed for the expression of the pre-meiotic/meiotic marker γH2AX, the epithelial somatic markers ERα, LHX9 and PITX2. **a** E12 wild-type. The right gonad, negative for PITX2 has a flat epithelium positive for LHX9 but negative for ERα. **b**, **c** Infected samples. PITX2 expression in the right gonad correlates with areas of cortex formation. **b** The right gonad is indistinguishable from the left; it has a well developed cortex whose somatic cells are double positive for LHX9 and ERα and contains γH2AX-positive germ cells. **c** The right gonad shows discrete epithelial areas with a developed cortex LHX9-ERα positive, containing clusters of meiotic germ cells; left-like areas are highlighted with a circle. L = Left gonad; R = right gonad. Size of bars = 100 μm.

data]. As it has been previously shown that PITX2 dosage affects organ morphogenesis [Kozlowski and Walter, 2000; Liu et al., 2001], it is conceivable that the reduced levels of PITX2 found at HH38 may have a different effect on RA signalling, compared to the effect seen at earlier stages. However, the putative late activation of RA signalling observed in the cortex suggests a more complex relationship between RA and L:R identity, and that RA signalling only needs to be temporarily downregulated in the epithelium at early stages of ovary development to allow the formation of the cortex.

It is unclear how PITX2 might control RA signalling. It may be that PITX2 regulates the expression of the genes involved in RA metabolism, as misexpression of *PITX2* on the right side caused down-regulation of *RALDH2* expression and misexpression of *PITX2*-engrailed on the left side caused upregulation of *RALDH2* expression [Ishimaru et al., 2008].

It has not been established that RA signalling is the only primary downstream target of PITX2 activity: for example, a signalling molecule expressed asymmetrically in the epithelium of the left undifferentiated gonad, and

which has been shown to be PITX2-dependent, is BMP7 [Hoshino et al., 2005; Guioli and Lovell-Badge, 2007], although the impact of these findings on BMP signalling and on asymmetry is unclear.

In addition, canonical WNT signalling may play a role in ovary development in birds. This is a major determinant of ovarian fate in mammals and is regulated via complex feedback interactions that include *Wnt4* and *Rspo1* [Zaytouni et al., 2011; Tevosian, 2013]. In the chicken, as in the mouse, these 2 molecules are upregulated in females at the time of gonadal differentiation, suggesting a conserved role in ovarian differentiation. Interestingly, in the chick, these proteins are mostly localised within the developing cortex of the left ovary [Smith et al., 2008b; Ayers et al., 2013]. Although, as yet, there is no direct evidence linking WNT signalling and the functional asymmetry of ovarian differentiation, it is known that WNT signalling and PITX2 are engaged in positive feedback regulation in many systems [Kioussi et al., 2002; Briata et al., 2003; Vadlamudi et al., 2005; Amen et al., 2007; Abu-Elmagd et al., 2010; Zacharias and Gage, 2010; Basu and Roy, 2013].

Finally, PITX2 may directly provide fine-tuning of genes involved in epithelial morphogenesis (RA sensitive or insensitive). Indeed *Pitx2* is a known regulator of proliferation through direct activation of specific growth control genes [Kioussi et al., 2002; Baek et al., 2003; Gherzi et al., 2010; Basu and Roy, 2013]. For example, a direct effect on *CD1* by PITX2 cannot be excluded as PITX2 has been shown to bind the *CD1* promoter in other tissues [Baek et al., 2003]. In addition, a number of studies indicate that PITX2 controls genes involved in the reorganisation of the cytoskeleton, affecting cell spreading, migration and cell-cell-adhesion [Wei and Adelstein, 2002; Campbell et al., 2012].

L:R Asymmetry and Sex (Gonadal) Determination

Our current view of gonadal sex determination is primarily based on work carried out on mammalian models. The so-called bipotential gonad is usually envisaged as a domain of competition between 2 opposing influences, and the initial point of gonadal sex determination is effectively the time when an imbalance is created in favour of one system. It is thought that the cell fate decision made within individual somatic cells is then coordinated to pattern the entire field according to the chosen pathway [Kim and Capel, 2006]. In general, it seems that the molecules that regulate implementation of the male and female differentiation programmes (e.g. SOX9, FOXL2) are conserved across vertebrates, while the primary triggers that initiate these programmes, are not. In mammals, the Y-chromosome gene *Sry* acts as a trigger for the activation of *Sox9*, the master regulator of Sertoli cell differentiation. Ovarian differentiation is less well understood, but it seems to involve more than 1 pathway acting in a cooperative manner, including FOXL2 and β-catenin regulatory networks. In the mouse, FOXL2 is only required after birth, whereas in some other mammals (e.g. goat) it is also essential for female sex determination [DeFalco and Capel, 2009; Veitia, 2010; Cutting et al., 2013; Sekido and Lovell-Badge, 2013; Tevosian, 2013].

In recent years it has become evident that, following the initial commitment to the female or male pathway, the gonads retain a plasticity and that even adult gonads may undergo extensive reprogramming upon the loss of key sex regulators. In the mouse, *Foxl2*/ER are required in the ovary to repress the male factor *Sox9* and to maintain female granulosa cell identity, while *Dmrt1* is required in the testis to repress *Foxl2* and to maintain testis identity, suggesting that the antagonism between *Foxl2* and *Dmrt1* is critical for the stability of the gonadal sex [Uhlenhaut et al., 2009; Matson et al., 2011].

In birds, it has long been established that the females are the heterogametic sex (ZW sex chromosomes) while the males are homogametic (ZZ); however, the mechanism of sex determination is still poorly understood. The involvement of a sex-specific primary trigger akin to the mammalian *Sry* gene [Koopman et al., 1991] would require a W-chromosome-encoded ovary-determining gene, but, to date, there is no evidence for the existence of such a factor. An alternative hypothesis proposes that the primary gonadal sex determination trigger is dependent on the level of expression of a Z-chromosome gene(s), with males (ZZ) having higher levels of expression than females (ZW) [reviewed in Clinton, 1998; Cutting et al., 2013]. It is known that the Z-chromosome *DMRT1* gene is expressed at higher levels in the male gonads than in the female gonads, from as early as HH25 [Smith et al., 1999]. Moreover, it has also been shown that down-regulation of *DMRT1* expression in the male genital ridges in ovo causes a degree of testis-to-ovary transformation, indicating that DMRT1 is required for testis determination [Smith et al., 2009; Chue and Smith, 2011]. These findings support the hypothesis of a Z-chromosome dosage-based mechanism of sex determination in birds and suggest that DMRT1 plays a key role in this process. However, it remains to be demonstrated that a threshold of *DMRT1* expression alone is sufficient to trigger testis differentiation

and so the involvement of other Z-linked (or even W-linked) factors cannot be excluded. In this context, it has recently been reported that the Z-chromosome gene hemogen is expressed at higher levels in the left and right medulla of male gonads than of female gonads between HH28 and HH35. Overexpression of this transcription factor in female embryos caused decreased FOXL2/P450arom expression and increased SOX9 expression compared to controls and a degree of female to male gonad sex reversal, suggesting that this gene could also play a role in testis determination [Nakata et al., 2013].

A study generating mixed-sex chimeric chickens recently demonstrated that avian cells have a cell-autonomous sex identity, and this could also support a dosage-based mechanism of gonadal sex determination [Zhao et al., 2010; Clinton et al., 2012]. It was found that individual male (ZZ) cells located in a developing ovary (ZW) were not incorporated into the aromatase-expressing medullary cords. However, if present in sufficient number, these cells differentiated into Sertoli cells and initiated sex-cord formation. Conversely, ZW cells in a developing testis responded by initiating ovarian development and expressing aromatase. Clearly the 'donor' cells in these mixed-sex chimeras can correctly interpret the host developmental signals for gonad differentiation, but respond in a donor-specific, cell-autonomous fashion. If the outcome of the developmental process that commits the gonad to a sex-specific pathway was simply a signal that initiates *DMRT1* expression from the Z chromosomes in both sexes, then male left and right medullary cells (ZZ) would automatically express twice as much *DMRT1* transcript as female cells (ZW). Higher levels of DMRT1 could then lead to the expression of *SOX9* and initiate Sertoli cell differentiation. In chicken, the activation of *SOX9* is observed between HH30–31 [Oreal et al., 1998; Moniot et al., 2008] and does coincide with the reorganisation of the primitive cords within the medulla into testis cords containing most of the germ cells. Conversely, the lower level of DMRT1 produced from the single Z chromosome in female cells would result in the activation of FOXL2 in the medulla, possibly leading to repression of *DMRT1* and male signals and to the promotion of female signals, including aromatase. Indeed, FOXL2 can activate the aromatase promoter in vitro [Pannetier et al., 2006; Wang et al., 2007; Fleming et al., 2010], and FOXL2 protein expression is female-specific and starts at HH28–29, just prior to the expression of aromatase [Govoroun et al., 2004; Pannetier et al., 2006; Ayers et al., 2013].

The synthesis of gonadal estrogens around the point of sex determination occurs in a large number of vertebrates, including some mammals, and for some of these species, it has been shown that this steroid is a major player in gonadal sex determination/differentiation. In this respect, the chicken is no exception [Shore and Shemesh, 1981; Scheib, 1983; Fadem and Tesoriero, 1986; Crews et al., 1991; Elbrecht and Smith, 1992; Matthiessen and Sumpter, 1998; Coveney et al., 2001; Quirke et al., 2001; Pieau and Dorizzi, 2004; Hudson et al., 2005; Pailhoux et al., 2005; Pettersson et al., 2006; Zha et al., 2008; Barske and Capel, 2010; Pask et al., 2010]. Indeed, using an aromatase inhibitor (fadrozole) to block estrogen synthesis in the female chick embryo leads to an up-regulation of *DMRT1* expression from the single Z chromosome, and to increased levels of SOX9 and decreased levels of FOXL2 in left and right medulla [Smith et al., 2003; Hudson et al., 2005]. Although fadrozole treatment almost invariably leads to the differentiation of a testis on the right side of female embryos, the fate of the left gonad is less clear-cut. The left gonad can present as a testis, an ovotestis or an ovary, presumably reflecting the effectiveness of estrogen inhibition. Intriguingly, on the left side it is not uncommon to find embryos with a masculinised medulla juxtaposed to a cortical structure [Vaillant et al., 2001a, b], suggesting that sex-specific differentiation of medulla and epithelium can, to some extent, be uncoupled at the time of gonadal determination. This reinforces the concept that, unlike the situation seen in the mouse, the chick medulla and epithelium are already quite distinct domains at the time of sex determination. Perhaps in some fadrozole-treated embryos, sufficient estrogen is produced to stimulate the initial differentiation of an ovarian cortex, but this is insufficient to prevent the formation of medullary testis cords along the male pathway, a scenario suggesting that estrogen signalling may act as both an antagonist of the male pathway and a promoter of ovarian differentiation.

In most vertebrates, the commitment to one sex results in the development of paired bilateral gonads indicating that, in most cases, the basic L:R asymmetry does not impact on the process of gonadal sex determination and gonadal development. Even in birds, the epithelial L:R asymmetry has little effect on testes differentiation, suggesting that the male pathway can either override or ignore differences between the right and left epithelia. In contrast, morphogenesis of the germinal epithelium is a central event in female gonadal development, and L:R asymmetry is clearly critical for implementation of the ovarian pathway in birds.

Our current understanding of the regulation of female gonadal asymmetry is summarised in figure 7.

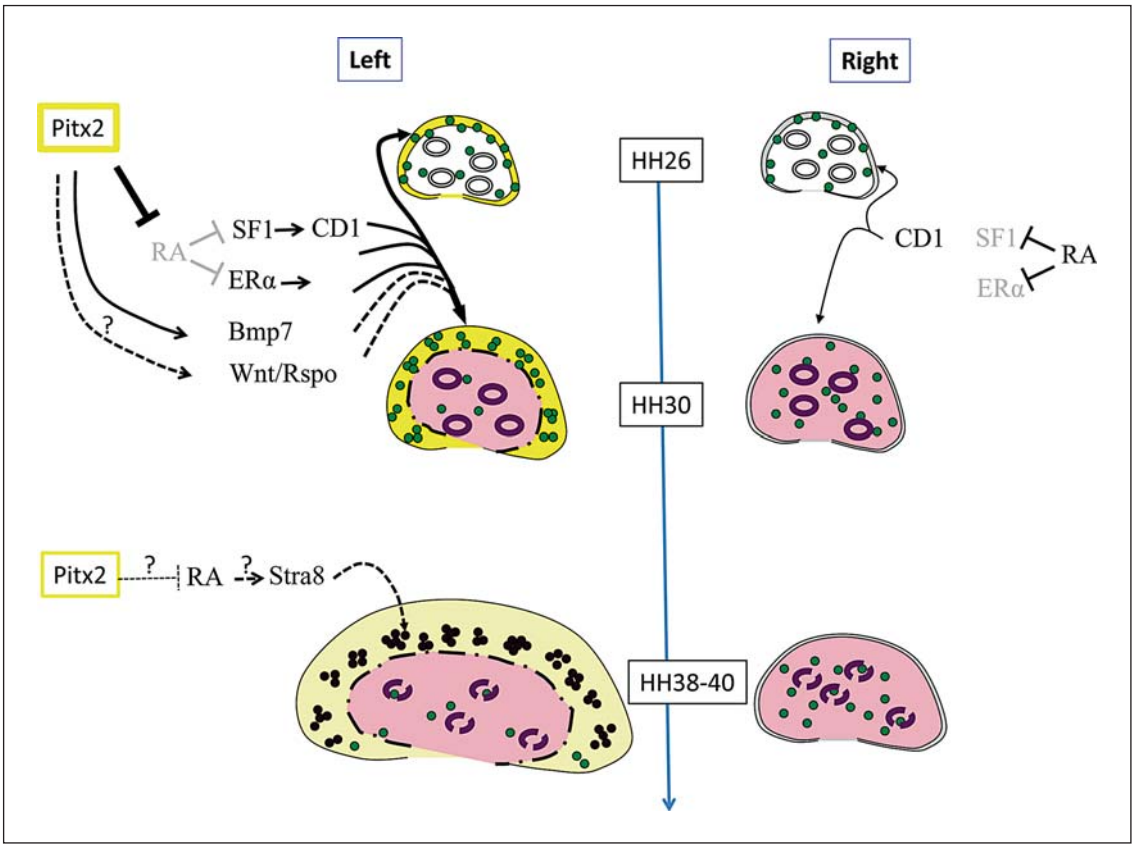


Fig. 7. Schematic illustrating the current understanding of asymmetrical ovarian development in the female chick embryo. Morphological changes in the developing left and right gonads. HH26: the epithelium is thicker in the left (in yellow) than in the right (in grey) gonad; the germ cells (green dots) migrating to the gonads localise close to the epithelium in both left and right gonad but they are more abundant in the left. HH30: right and left medullary cords (dark pink circles) start to express P450 aromatase and to produce estrogen; on the right, the epithelium changes polarity and the germ cells become scattered in the medulla; on the left, the epithelium starts to stratify and most germ cells stay in the epithelium layer. By HH38 both left and right ovary medullary cords have become vacuolated to form lacunae (dark pink open circles) and contain some of the medullary germ cells; no further development is visible in the right ovary; the left ovary has developed a stratified cortex containing most germ cells committed to meiosis (black dots). Current model of the molecular network involved in the differentiation of the ovarian cortex and affected by the L:R asymmetry pathway via PITX2: In the left epithelium PITX2 promotes the expression of *ERα* and *SF1* by blocking RA signalling (black lines). This is thought to result in higher proliferation of the epithelium and ability to respond to E2. Other signalling pathways may also be involved including WNT and BMP. By HH38, although *PITX2* is still expressed, RA signalling in the cortex is active and is linked to *STRA8* activation and initiation of meiosis. Dotted lines indicate possible interactions.

Discussion

There are various asymmetrical features of gonadal development in birds; some are present in both sexes while others are restricted to the female. Prior to the point of gonadal sex determination, 2 morphological L:R asymmetries are common to males and females: first, a greater proportion of the circulating PGCs colonise the left gonad than the right, and second, the epithelial layer covering the left gonad is thicker than that covering the right gonad. After gonadal sex determination, a further asymmetry is restricted to females: only the gonad on the left side develops a cortex and will mature into a fully functional ovary. As regards the right ovary, we and others have routinely described this organ as regressing, but this may be somewhat misleading as it clearly increases in size and differentiates to the extent of generating roughly equivalent levels of aromatase to that produced by the left ovary. The 2 basic roles of an ovary are (a) to nurture and deliver ova, and (b) to function as an endocrine organ.

Perhaps in birds, the left ovary performs both roles while the right is limited to acting as an endocrine organ during embryonic development.

Although one obvious possibility is that the development of a reproductive system with only 1 ovary and 1 oviduct is an adaptation to flight, the evolution of this phenomenon is not clear. In most instances, the ability to fly is clearly associated with 1 ovary/1 oviduct – for example, the majority of modern flying birds have only 1 ovary and 1 oviduct, and bird fossils from the Cretaceous period [125 million years ago (mya)] have a single ovary on the left side, while the non-flying dinosaur ancestors of birds had 2 oviducts and (it is assumed) 2 ovaries [Zheng et al., 2013]. However, in other instances this association is not clear-cut. Amongst the group known as the flightless birds (the ratites), that diverged from modern birds between 60–100 mya [Höhn, 1947; Lofts and Murton, 1973; Cooper and Penny, 1997; Clarke et al., 2005; Harshman et al., 2008] and lost the ability to fly, the kiwi has 2 ovaries while all the other ratites have only one [Kinsky, 1971]. Even in some modern birds such as certain types of birds of prey (e.g. Falconiformes), which appeared only around 10 mya, the incidence of paired ovaries is high [Gunn, 1912; Crew, 1931; Shaw, 1938; Kinsky, 1971; Walter, 1979].

This raises the question as to why would a single ovary/oviduct be a useful adaptation to flight. The most popular theory is that this is an issue of weight – rather along the lines of an airline 'baggage allowance' – but relating to the ovary with its hierarchy of large maturing follicles as opposed to the weight of the egg [Zheng et al., 2013]. However, most birds have only a short breeding season, and outwith this period the ovaries are small and quiescent, and do not contain mature follicles. Gonadal weight would only be a serious issue during the breeding season and would also apply to males, as it is not only the single ovary in females but also both (internal) testes in males that increase dramatically in size due to the production of gametes [Höhn, 1947; Lofts and Murton, 1973].

An alternative theory to account for the adoption of a single ovary/oviduct system relates to the fragility of the egg during the final stage of development. Between ovulation and lay, the ovum spends around a day in the oviduct acquiring an eggshell. During this period, the egg is clearly susceptible to physical damage, and it has been suggested that the simultaneous presence of 2 or more shelled eggs in close proximity within the abdomen would lead to a reduction in survival rate [Walter, 1979]. Perhaps this susceptibility to damage led to birds adopting a reproductive system with a single oviduct and this in turn led to the retention of only 1 ovary – in a system with 1 oviduct and 2 ovaries, the production of mature follicles would have to be very tightly coordinated. It is noteworthy that even birds with paired ovaries tend to have only 1 oviduct, typically on the left [Kinsky, 1971]. Of course, the evolution of the shelled egg containing all the materials necessary for complete embryonic development requires that very large ova are produced, and so perhaps it is a combination of space restrictions and egg fragility that led to the development of a single ovary/oviduct system in (most) birds, rather than a question of weight. In this context, it is interesting to note that another egg-laying (non-flying) animal, the platypus, also has only a single functional ovary on the left side [Grützner et al., 2008].

The final question concerns the relationship between the formation of 1 ovary on the left side and the basic system of L:R patterning in vertebrates. It is possible that, in the majority of vertebrates, L:R patterning inhibits ovary development on the right side and/or promotes ovary development only on the left side, and that this asymmetry is repressed to generate paired ovaries – along the lines reported to allow bilateral somite development [Vermot and Pourquie, 2005; Duester, 2007]. An alternative possibility is that the basic L:R patterning system imposes only minor difference between the gonads in all vertebrates, and that most birds have an additional mechanism to promote ovary development on the left side and/or inhibit ovary development on the right side. This could again involve RA signalling as discussed.

The question remains unresolved and further functional studies on the role of the epithelial molecular asymmetries are required. The analysis of *PITX2* expression and RA signalling in the birds with a high incidence of 2 ovaries would be particularly informative. This should establish the point in the cascade of events in gonad development where asymmetry is overcome in birds with paired ovaries, and potentially shed new light on how the basic L:R asymmetry pathway intersects with ovarian differentiation.

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